Biochimica et Biophysica Acta 1833 (2013) 80–89

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Glutathionylation of UCP2 sensitizes drug resistant leukemia cells to chemotherapeutics

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ARTICLE INFO

Article history:
Received 5 September 2012
Received in revised form 3 October 2012
Accepted 4 October 2012
Available online 12 October 2012

Keywords:
Uncoupling protein-2
Glutathionylation
Drug resistance
Proton leak
Chemotherapy
Mitochondria

ABSTRACT

Uncoupling protein-2 (UCP2) is used by cells to control reactive oxygen species (ROS) production by mitochondria. This ability depends on the glutathionylation state of UCP2. UCP2 is often overexpressed in drug resistant cancer cells and therein controls cell ROS levels and limits drug toxicity. With our recent observation that glutathionylation deactivates proton leak through UCP2, we decided to test if diamide, a glutathionylation catalyst, can sensitize drug resistant cells to chemotherapeutic agents. Using drug sensitive HL-60 cells and the drug resistant HL-60 subline, Mx2, we show that chemical induction of glutathionylation selectively deactivates proton leak through UCP2 in Mx2 cells. Chemical glutathionylation of UCP2 disables chemoresistance in the Mx2 cells. Exposure to 200 μM diamide led to a significant increase in Mx2 cell death that was augmented when cells were exposed to either menadione or the anthracycline doxorubicin. Diamide also sensitized Mx2 cells to a number of other chemotherapeutics. Proton leak through UCP2 contributed significantly to the energetics of the Mx2 cells. Knockdown of UCP2 led to a significant decrease in both resting and state 4 (i.e., proton leak-dependent) respiration (~43% and 62%, respectively) in Mx2 cells. Similarly diamide inhibited proton leak-dependent respiration by ~64%. In contrast, diamide had very little effect on proton leak in HL-60 cells. Collectively, our observations indicate that manipulation of UCP2 glutathionylation status can serve as a therapeutic strategy for cancer treatment.

1. Introduction

Aerobic production of ATP is reliant on molecular oxygen (O_2) and the formation of an electrochemical potential ($\Delta\Psi_m$) across the mitochondrial inner membrane (MIM) [1]. In this process, electrons from substrates are transferred through the respiratory complexes to O_2 and this is coupled to the efflux protons into the intermembrane space, thus establishing the $\Delta\Psi_m$. This stored form of energy is then tapped by ATP synthase to generate ATP, a process termed coupled respiration. However, protons pumped into the intermembrane space can by-pass ATP synthase and re-enter the matrix without generating ATP and this is referred to as uncoupled respiration [2]. The Gibbs free energy stored in $\Delta\Psi_m$ is also used for the uptake of organic acids, cations/anions, and proteins. The uncoupling proteins (UCPs) are thought to translocate protons back into the matrix without coupling it to the co-import of other molecules [3]. It is important to point out though that other reports have shown that UCPs 1–3 transport metabolites (e.g., lipid hydroperoxides) and calcium [4,5]. Whether or not these putative transport functions require co-import of protons remains unclear. However, despite the controversy surrounding UCPs, there are a number of studies that attribute the ROS-quenching effects of UCPs to proton leak [6–8]. Indeed, proton leak through the UCPs is biologically important. For instance, proton leak through UCP1, which is exclusively expressed in brown fat, is required for adaptive thermogenesis [9]. However, leak through the other UCPs, UCP2 and UCP3, is not used for thermogenesis but to control mitochondrial reactive oxygen species (ROS) emission [10]. A higher $\Delta\Psi_m$ increases the likelihood that electrons will "spin-off" mainly from complexes I and III and produce ROS [11,12]. For this reason, mitochondria are considered to be one of the main sources of cellular ROS. The ability to control ROS through proton leak is considered the first line of defense against oxidative stress and is associated with increased cell survival [13].

UCP2 plays important physiological roles ranging from control of insulin release to satiety signaling and cell proliferation [14–16]. The ability of UCP2 to participate in so many physiological functions has been associated with its putative inducible proton leak mechanism, which in turn affects ROS signaling cascades in a variety of cell types [2]. It has been established that UCP2 proton leak diminishes mitochondrial ROS production, which subsequently regulates signaling cascades [17,18]. Leak is dependent on ROS levels and reversible glutathionylation, a posttranslational modification involving the formation of a disulfide between glutathione and a protein thiol [19,20]. Indeed, induction of glutathionylation with diamide in thymocytes deactivates proton leak in a UCP2-specific manner [19]. Further, ROS deglutathionylates UCP3,

Abbreviations: UCP2, uncoupling protein-2; ROS, reactive oxygen species; $\Delta\Psi_m$, electrochemical potential; MIM, mitochondrial inner membrane

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http://dx.doi.org/10.1016/j.bbamcr.2012.10.006

a homologue of UCP2, which activates proton leak. Leak through UCP3 is easily reversed by diamide treatment [19]. Aside from its important signaling functions, leak through UCP2 is required to keep ROS levels in check. Loss of UCP2 function sensitizes cells to oxidative stress [21,22]. Likewise, overexpression provides enhanced protection from ROS-producing molecules [23]. Hence, UCP2 functions to control mitochondrial ROS emission through a proton leak mechanism.

Drug resistant cancer cells use many mechanisms to render chemotherapeutic agents innocuous. One such mechanism is increased UCP2 expression [24,25]. By increasing UCP2 expression, drug resistant cancer cells increase proton leak which keeps cellular ROS levels within tolerable limits, even when exposed to ROS-generating chemotherapeutics [21,23,25–27]. Various cancer cell types, including breast cancer cells, leukemia cells, human colon cancer cells, thyroid tumours, and hepatomas, use UCP2 to fend off against the toxic effects of chemotherapeutic agents [28–32]. With our recent observation that UCP2 is modulated by glutathionylation, we set out to determine if pharmacological glutathionylation of UCP2 with diamide, a glutathionylation catalyst, sensitizes drug resistant promyelocytic leukemia cells (HL-60/Mx2 or Mx2) to chemotherapeutics. Unlike the drug sensitive parent line, drug resistant Mx2 cells display higher rates of aerobic respiration, fatty acid oxidation, and glucose metabolism [25,26]. Mx2 cells also have normal cellular ROS levels and are highly resistant to the toxicity of a broad range of chemotherapeutics [26,33]. These sharp deviations in cell physiology are mostly attributed to the amplification of UCP2 expression. Indeed, in comparison to other antioxidant proteins, UCP2 is heavily expressed in Mx2 cells, indicating the drug resistance of this cell line is highly dependent on UCP2 [26]. Here we report that acute treatment of Mx2 cells with diamide led to a robust decrease in UCP2-dependent proton leak. This effect was absent in the drug sensitive promyelocytic leukemia parent cell line (HL-60). The diamide-mediated decrease in proton leak augmented the sensitivity of the Mx2 cells to a number of different chemotherapeutic agents such as the anthracycline, doxorubicin, and the ROS-generating menaquinone, menadione. Collectively, our results show that targeted inhibition of UCP2 function by glutathionylation can serve as a therapeutic approach to kill cancer cells.

2. Materials and methods

2.1. Cell lines and treatment

The drug sensitive human acute promyelocytic leukemia (APL) suspension cell line HL-60 and drug resistant subline Mx2 were obtained from American Type Culture Collection (ATCC, Manassas, VA). Authentication regarding sensitivity and resistance to chemotherapeutics was performed by ATCC prior to shipping. The cells were routinely cultured in high glucose Dulbecco's modified Eagle's medium (DMEM containing 25 mM glucose, 4 mM glutamine, 1 mM pyruvate, Invitrogen, Burlington, ON) supplemented with 20% (v/v) fetal bovine serum (FBS, Invitrogen, Burlington, ON) and 2% (v/v) antibiotic–antimycotic (A.A., Invitrogen, Burlington, ON). Every 2 days the media was changed and every 4 days the cells were passaged and reseeded at 1×10^5 cells/mL in a final volume of 13 mL. Cells were grown for 4–5 days before each experiment. Cell number and viability were determined using the trypan blue exclusion assay. Cells were cultured for up to 15–20 passages, at which point they were discarded. For diamide treatments, following 4 days of growth, cells were treated with diamide (diluted in growth medium, 0–500 μM), incubated for 30 min at 37 °C, and used for experimentation. To test the effect of diamide on drug sensitivity, following diamide treatment, cells were centrifuged at 200×*g* and then incubated for 24 h in growth medium containing menadione (0–100 μM) or doxorubicin hydrochloride (0–0.5 μM) [26].

For UCP2 knockdown, cells were grown for 2 days and then treated with Polybrene (2 μg/mL, Santa Cruz Biotechnologies) with either UCP2 shRNA (shUCP2; Santa Cruz Biotechnologies) or scrambled (control, shCtl; Santa Cruz Biotechnologies) shRNA lentiviral particles (5000 infectious viral particles (IFU)/mL, Santa Cruz Biotechnologies) for 48 h. Cultures were then re-supplemented with fresh medium devoid of lentiviral particles and Polybrene, and then incubated for an additional 24 h in a medium including puromycin (1 μg/mL, Santa Cruz). Transduced cells were then treated with diamide and isolated for experimentation.

2.2. Cell survival assays

Cell survival was determined following diamide treatment using a propidium iodide (PI) assay [26]. Cells were washed twice in washing buffer (PBS + 10 mM glucose) followed by a 10 min incubation in PI (100 μg/mL, Sigma, St. Louis, MO) at room temperature. The PI solution was then removed and cells were washed in washing buffer before being placed in a 96-well plate in triplicates. Cells exposed to 5 mM H_2O_2 served as the control. Excitation was measured at 485 nm and emission at 645 nm using a microplate reader (SynergyMX, BioTek, Winooski, VT). PI results were normalized to cell number.

2.3. Trypan blue exclusion assays

Trypan blue exclusion assays were conducted as described in [26]. An aliquot of the cell suspension was diluted in trypan blue solution (Invitrogen, Burlington, Ontario) and cell viability was determined using the Countess Cell Counter according to the manufacturer's instructions (Invitrogen, Burlington, ON).

2.4. Measurement of cellular ROS levels

2′,7′-Dichlorodihydrofluorescin diacetate (H_2-DCFDA) was used to measure total cellular ROS levels [26] and MitoSOX was used to measure mitochondrial superoxide levels. For H_2-DCFDA, following diamide treatment, cells were centrifuged at 200×*g* for 5 min, washed in 1 mL washing buffer and then incubated in growth media containing H_2-DCFDA (20 μM). H_2-DCFDA was removed and the sample was washed twice in washing buffer before being placed in a 96-well plate in triplicates. Cells exposed to 5 mM H_2O_2 served as the control. To measure mitochondrial superoxide cells were pre-loaded with MitoSOX (40 μM final concentration, 10 min incubation) and then incubated in diamide for an additional 30 min at 37 °C. Cells were then washed twice in washing buffer before being placed in a 96-well plate in triplicates. Cells exposed to 1 mM paraquat (PQ; superoxide-producing bipyridine molecule) served as the control. A 10 μL aliquot of the sample was removed and cell number and viability were determined using the trypan blue exclusion assay following diamide exposure for all assays. Excitation was measured at 480 nm and emission at 528 nm for H_2-DCFDA and 510 nm/580 nm for MitoSOX using a microplate reader (SynergyMX, BioTek, Winooski, VT). ROS levels were normalized to cell number.

2.5. HPLC analysis of cellular glutathione pools

Glutathione quantification was conducted as described in [34]. Cells treated with or without 200 μM diamide were washed in PBS before being resuspended in 100 μL of 0.5% perchloric acid, vortexed, and incubated on ice for 10 min. The resulting precipitate was removed by centrifugation at 12000×*g* for 10 min at 4 °C. For GSH/GSSG determinations, the supernatant was extracted and used. For determinations of total glutathione associated with the proteome, the protein pellet was resuspended in 100μL of 10 M sodium hydroxide and incubated for 10 min at room temperature to hydrolyze thiol bonds between proteins and glutathione. Samples were then centrifuged at 12000×*g* for 10 min at 4 °C and the supernatant was collected for analysis. Samples were injected into an Agilent HPLC system equipped with a Pursuit C18 150×4.6 nm, 5 μL column (Agilent Technologies). The mobile phase consisted of ddH_2O + 0.1% trifluoroacetic acid (TFA), pH 2.0: CH_3OH

– 90:10. Detection was performed at 215 nm, the flow rate was set to 1.0 mL/min and samples were run for 15 min each. Retention times of reduced (GSH) and oxidized (GSSG) glutathione were confirmed by injecting known standards. GSH and GSSG were quantified using Agilent Chemstation software. All values were normalized to cell protein content.

2.6. Measurement of oxygen consumption

Cells were counted and diluted to 13.3×10^6 cells for HL-60 and 3.3×10^6 cells for Mx2 and then treated with and without 200 μM diamide. Different amounts of cells were used since HL-60 cells are not as aerobically active as Mx2 cells [25]. Following treatment, cells were washed in washing buffer before being placed on ice. One milliliter of reaction buffer (106 mM NaCl, 0.41 mM $MgCl_2$, 25 mM Na_2HPO_4, 5 mM KCl, 10 mM glucose, pH 7.0) was pre-heated to 37 °C before measurements were performed. Cell suspensions were then placed in a temperature-regulated Clark-type electrode chamber (Oxytherm; Hansatech Instruments Ltd, Norfolk England). Once resting respiration was determined, state 4 respiration rates were assessed by injecting oligomycin (final concentration 10 μg/mL) into the electrode chamber. Oxygen consumption was measured for 5–15 min at 37 °C. Oxygen consumption was normalized to cell number.

2.7. Chemotherapeutics screen

Chemotherapeutic screens were performed using the Biolog assay kit (PM-M14, Biolog, Hayward, CA) according to the manufacturer's instructions. Cells treated with and without 200 μM diamide were washed in washing buffer and then seeded at 4×10^4 cells/mL in 96-welled Biolog plates containing serum-free growth media. Cells were incubated in the presence of the chemotherapeutic drugs for 24 h at 37 °C. Changes in cell viability were determined by adding 10μL of Redox Dye Mix B (Biolog, Hayward, CA) directly to each well. Cells were then incubated for an additional 24 h. Absorbance was measured at 590 nm using a microplate reader (SynergyMX, BioTek, Winooski, VT).

2.8. BioGEE pulldown and immunoblot analysis

Glutathionylation state of UCP2 was tested as described in [19]. Cells were grown to 50% confluency and then incubated for 1 h in serum free DMEM containing 1 mM biotinylated glutathione ethyl ester (BioGEE). Following the incubation, cells were isolated by centrifugation, washed once in washing buffer, and then lysed in RIPA buffer containing 25 mM N-ethylmaleimide (NEM). Cell lysate was then incubated overnight at 4 °C under constant agitation in streptavidin beads to elute BioGEE-tagged proteins. Beads were first isolated by centrifugation ($200\times g$ for 5 min) and the resulting pellet was washed once with PBS. Note that the supernatant from the centrifugation (SN fraction) was kept for immunoblot analysis. The beads were then treated with 4 M urea (in PBS pH 7.6) to dissociate BioGEE-tagged proteins from the beads. The sample was then centrifuged to remove the beads and the resulting supernatant (pull down fraction; PD) was kept for immunoblot analysis.

For immunoblot, samples were diluted to 1 mg/mL in Laemmli buffer and 30 μg of cell lysate was electrophoresed on a 12% isocratic SDS-gel. For BioGEE samples, samples were diluted to 2–4 mg/mL and 60 μg of protein was loaded. Transfers to nitrocellulose membranes were performed at room temperature at a voltage of 100 V. Following blocking, membranes were incubated for 24 h at 4 °C with primary antibodies directed against UCP2 (1/500, Abcam) or succinate dehydrogenase (SDH, 1/2000, Santa Cruz). For detection of UCP2 elution, Ponceau staining of membranes served as the loading control. Membranes were then incubated for 1 h at room temperature with the requisite horseradish peroxidase-conjugated secondary antibody (anti-rabbit, anti-mouse, or anti-goat, 1/2000, Santa Cruz). Blots were visualized using enhanced chemiluminescent substrate (ECL kit, Thermo Scientific).

2.9. Statistical analysis

Statistical analysis was performed using either paired one-tailed Student's *t* test or one-way or two-way ANOVA with post-hoc Fisher tests to determine statistical differences in means (Statview software, SAS Institute Inc., USA). $p\leq0.05$ was considered to be significant. All values were expressed as mean ± SEM.

3. Results and discussion

3.1. Impact of diamide on HL-60 and Mx2 cell viability

The fact that many drug resistant cancer cells express high amounts of UCP2 indicates that this protein could serve as a potential target for enhancing the effectiveness of pharmaceuticals. Inhibition of leak through UCP2 using genipin or the highly effective chromane compound sensitizes drug resistant cells to chemotherapeutics [21,26,27,35]. Overexpression of UCP2 on the other hand renders the ROS-generating properties of the chemotherapeutic gemcitabine innocuous [35]. With our recent observations that UCP2 can be deactivated by diamide-mediated glutathionylation, we set out to determine if induction of UCP2 glutathionylation with diamide sensitized drug resistant cells to chemotherapeutics. First, we performed dose response analysis to identify the appropriate concentration of diamide to use for our studies. Drug sensitive HL-60 cells and the drug resistant HL-60 subline, Mx2, were exposed to different concentrations of diamide (0–500 μM) for 30 min and then tested for amount of cell death using propidium iodide (PI). Cells were also exposed to 5 mM H_2O_2 for 30 min to induce maximum cell death. In HL-60 cells diamide treatment at all concentrations did not increase PI fluorescence relative to control (Fig. 1A). Mx2 cells responded differently to diamide treatment. Exposure to 500 μM led to an increase in cell death (Fig. 1A). Prolonged exposure to high concentrations of diamide is well known to induce cell death through the activation of the mitochondrial permeability transition pore (MPTP) which, once activated, depolarizes the MIM, thereby activating the intrinsic apoptotic signaling cascade [36,37]. However, our group has previously established a short incubation (10–60 min) with diamide concentrations below 500 μM can be used to test glutathionylation effects on metabolism without disrupting cell homeostasis [19,34]. Since glutathionylation deactivates UCP2, we decided to test if diamide treatment altered total cellular ROS (H_2-DCFDA) and mitochondrial ROS (MitoSOX). H_2-DCFDA assays revealed no changes in total cell ROS levels in either the HL-60 or Mx2 cells (Fig. 1B). H_2-DCFDA is non-specific, can be reduced by redox active metals and molecules, and can auto-catalyze ROS formation [38]. Further, leak through UCP2 is required to control mitochondrial ROS production and also protects cancer cells from ROS-generating molecules [24]. Thus, we decided to determine if diamide treatment specifically alters mitochondrial superoxide levels. Diamide treatment (0–500 μM) did not alter mitochondrial superoxide levels in HL-60 cells (Fig. 1C). Exposure of Mx2 cells to 200 μM diamide led to a small but significant increase in mitochondrial superoxide levels (Fig. 1C). This increase is most likely associated with the diamide-mediated glutathionylation of UCP2. Exposure to higher amounts of diamide (500 μM) led to further increases in mitochondrial superoxide. Mx2 cells also displayed much higher mitochondrial superoxide levels than the drug-sensitive HL-60 cells. Mx2 cells do have more mitochondria and these drug resistant cells are more aerobically active, which may account for the order of magnitude higher mitochondrial superoxide [25]. The higher mitochondrial superoxide levels in Mx2 cells could also explain why drug-resistant cells have higher levels of UCP2 protein. It is worthy to note that the remaining experiments were conducted on cells exposed to 200 μM diamide since this concentration

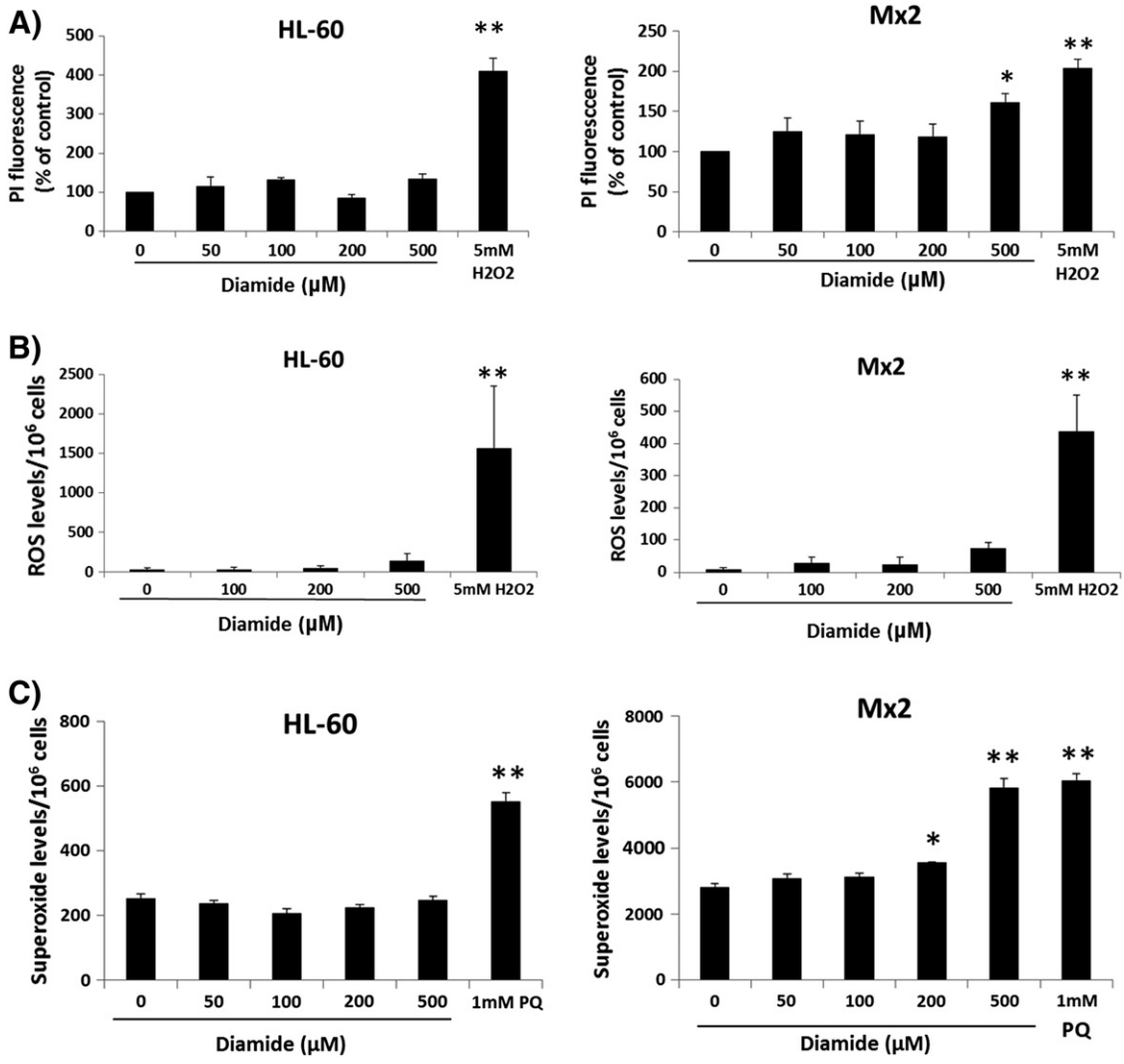

Fig. 1. Impact of diamide on HL-60 and Mx2 cell viability. Cells were exposed to diamide (0–500 μM) for 30 min and then tested for cell viability and cellular ROS levels. Exposure to 5 mM H_2O_2 or 1 mM PQ served as the control. (A) Diamide treatment increases Mx2 cell death. Following exposure to diamide, amount of cell death was measured by PI assay. Fluorescence was corrected for cell number and expressed as a percent of control. (B) The increase in diamide-induced Mx2 cell death is not associated with changes in total cell ROS levels. Amount of cell ROS was measured using H_2-DCFDA. (C) Diamide treatment increases mitochondrial superoxide levels in Mx2 cells but not HL-60 cells. Superoxide levels were measured using MitoSOX. All values were normalized to cell number. $n = 4$, 1-way ANOVA with post-hoc Fisher test. * corresponds to $p \leq 0.05$, ** corresponds to $p \leq 0.01$. Statistical significance was determined by comparing treated cells to control cells.

induced a small but significant increase in mitochondrial superoxide levels in Mx2 cells.

3.2. Effect of diamide on cellular glutathione pools

Diamide is known to alter or deplete cell glutathione, which could account for the increased amount of Mx2 cell death [39]. Indeed, survival of drug resistant cells depends to a large extent on the ROS quenching and xenobiotic eliminating capabilities of glutathione. Hence, we decided to measure the absolute levels of reduced and oxidized forms of glutathione (GSH and GSSG, respectively), as well as the GSH/GSSG ratio and the amount of glutathione associated with the cellular proteome. GSH is the dominant form of glutathione in the cell. This is maintained by a balance between reduction of GSSG back to GSH following ROS quenching and glutathione biosynthesis pathways [40]. However, in cancer cells the glutathione pool can range from highly reduced to extremely oxidized and this is dependent on tissue environment, nutrient status, O_2 availability, and the amount of ROS being produced. For instance, drug resistant cells and advanced cancers can have a highly oxidized glutathione pool which may be the result of ROS sequestration [41,42]. Absolute GSH levels were significantly lower in Mx2 cells in comparison to their drug sensitive counterpart (Fig. 2A). On the other hand GSSG levels were higher in Mx2 cells, indicating an increased propensity to oxidize GSH to GSSG. There was no observable difference in the total amount of free glutathione (GSH + GSSG) between the HL-60 and Mx2 cells, indicating no increase in glutathione biosynthesis in the drug resistant cells. Using the absolute GSH and GSSG levels, we also calculated the relative GSH/GSSG in HL-60 and Mx2 cells. The redox state of the GSH/GSSG pair can have a profound impact on cellular physiology and is highly variable in cancer cells. GSH/GSSG was more reduced in HL-60 than Mx2 cells (~25 vs. ~10) (Fig. 2A). Kawiak et al. previously showed that the GSH/GSSG is more oxidized in Mx2 cells [42]. However, in that study the authors calculated the GSH/GSSG in HL-60 and Mx2 cells to be ~12 and ~10, respectively. We also measured the total amount of glutathione associated with the cellular proteome. GSH can conjugate to available protein thiols forming protein glutathione mixed disulfides, a process called glutathionylation. Glutathionylation is a tightly regulated process and is enzymatically mediated mostly by glutaredoxins (Grx), and to a lesser extent sulfiredoxins and glutathione S-transferases [20]. However, a highly oxidized GSH/GSSG can lead to the spontaneous glutathionylation of proteins [43]. Non-specific glutathionylation reactions are associated

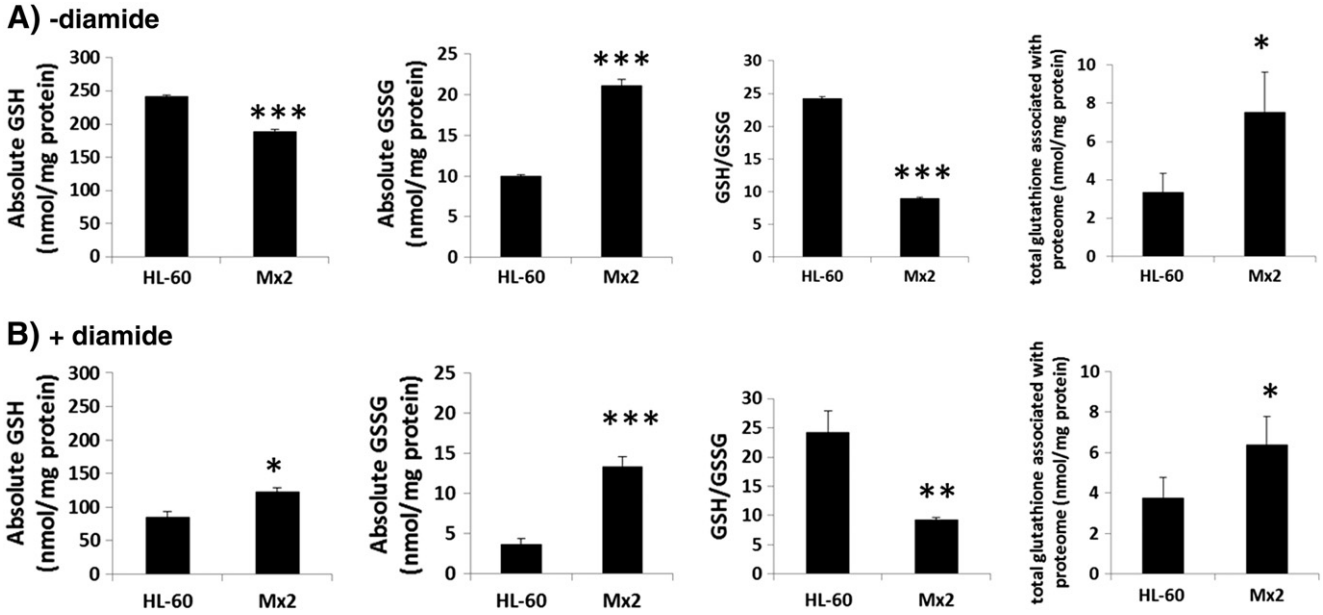

Fig. 2. Measurement of glutathione pools in HL-60 and Mx2 cells treated or untreated with diamide. Cells were incubated for 30 min in the presence or absence of 200 μM diamide. Gluathione was then extracted and the amount of GSH and GSSG was analyzed by HPLC. The GSH/GSSG was calculated based on the absolute GSH and GSSG levels. The amount of glutathione associated with cellular protein was also examined (see Materials and methods). (A) Mx2 cells have a more oxidized glutathione pool and have more protein glutathione mixed disulfides than HL-60 cells not treated with diamide. (B) Measurement of cellular glutathione and protein glutathione mixed disulfides in HL-60 and Mx2 cells treated with diamide. $n=3$, Student's t test. * corresponds to $p\leq 0.05$, ** corresponds to $p\leq 0.01$.

with various pathologies including heart disease, neurological disorders, and cancer [44]. Mx2 cells had more glutathione associated with the proteome than HL-60 cells (Fig. 2A).

Pre-treatment of HL-60 and Mx2 cells with 200 μM diamide induced a decrease in the absolute levels of GSH and GSSG (Fig. 2B). However, GSH and GSSG levels were significantly lower and higher, respectively, in Mx2 cells. While GSH/GSSG was still more oxidized in Mx2 cells, diamide (a glutathionylation catalyst) did not alter GSH/GSSG in either cell type (Fig. 2B). Intriguingly, diamide treatment did not cause detectable increases overall in the amount of glutathione protein conjugates. It is important to point out that concentrations of diamide around 100–200 μM do not induce hyper-glutathionylation [19]. Higher amounts (≥500 μM) are required to induce overt increases in the total amount of protein-glutathione adducts. This could account for the lack of total change in the glutathionylation state of proteins in each cell type. The amount of glutathione associated with cell protein was, however, still significantly higher in Mx2 cells (Fig. 2B). As expected though, diamide did partially deplete cell glutathione (both GSH and GSSG) in both HL-60 and Mx2 cells. Thus, diamide-mediated depletion of glutathione can be excluded as the reason for Mx2 cell death. Hence, we can conclude that Mx2 cells have a more oxidized glutathione pool and a more glutathionylated proteome. It is important to point out that cellular GSH concentration ranges from 5 to 10 mM. In our study diamide depleted GSH by ~50%, which would still leave mM amounts of GSH in the cell and would still be enough to quench ROS or detoxify xenobiotics. In addition, depletion of cell glutathione with butathione sulfoximine does not increase baseline cell ROS levels [19]. Moreover, the diamide-mediated increase in Mx2 cell death was not due to interference with cellular glutathione pools and is thus most likely associated with alterations in the glutathionylation of specific proteins.

3.3. Diamide treatment inhibits proton leak in Mx2 cells only

Given that UCP2 is highly expressed in drug resistant cancer cells, we set out to determine if diamide can inhibit proton leak in Mx2 cells. HL-60 and Mx2 cells were pre-treated with or without diamide for 30 min and then tested for O_2 consumption under resting and oligomycin-induced state 4 (proton leak or non-phosphorylating) respiratory conditions. To ensure total deactivation of ATP synthase, we used saturating amounts of oligomycin (10 μg/mL). Following diamide treatment, a trend for an increase in resting respiration was observed in the HL-60 cells; however, this did not reach significance (Fig. 3A). Diamide had no impact on oligomycin-induced state 4 respiration (Fig. 3A). A trend for increased resting respiration was also observed in the Mx2 cells (Fig. 3B). However, diamide treatment did induce a significant decrease in proton leak-dependent respiration in the Mx2 cells (Fig. 3B). In fact, diamide pre-treatment decreased leak by ~64% in Mx2 cells. It is important to point out that Mx2 cells also had higher resting O_2 consumption rates than their drug sensitive counterpart (~80% higher). This observation is consistent with our previous work showing that Mx2 cells are more aerobically active than HL-60 cells [25,26]. In contrast, leak was not affected in the drug sensitive HL-60 cells. The trend for an increase in resting respiration following diamide treatment prompted us to test the effect of higher diamide concentrations on resting respiration in HL-60 and Mx2 cells. Resting respiration tended to increase following titration of diamide up to 200 μM but this did not reach significance (Fig. 3C). Further increases in diamide (500 μM and 800 μM) led to a progressive suppression of respiration in both HL-60 and Mx2 cells (Fig. 3C). Diamide at low concentrations is known to stimulate respiration in certain cell types but in our case 200 μM diamide did not lead to a significant increase in respiration [45].

We next tested the glutathionylation state of UCP2. HL-60 and Mx2 cells were treated with biotinylated glutathione ethyl ester (BioGEE), which tags vacant glutathionylation sites on proteins with biotin. A more deglutathionylated protein will bind more BioGEE providing an index for protein glutathionylation state. More BioGEE-tagged UCP2 was eluted from Mx2 than HL-60 cells (Fig. 3D). It is important to remember that Mx2 cells overexpress UCP2 and rely on UCP2 to maintain low cell ROS levels even when exposed to pharmacological agents that induce oxidative stress [26].

3.4. Diamide effect on proton leak respiration in Mx2 cells is due to inhibition of UCP2

Our observation that diamide inhibits proton leak in Mx2 cells prompted us to determine if this was due to UCP2. We subsequently

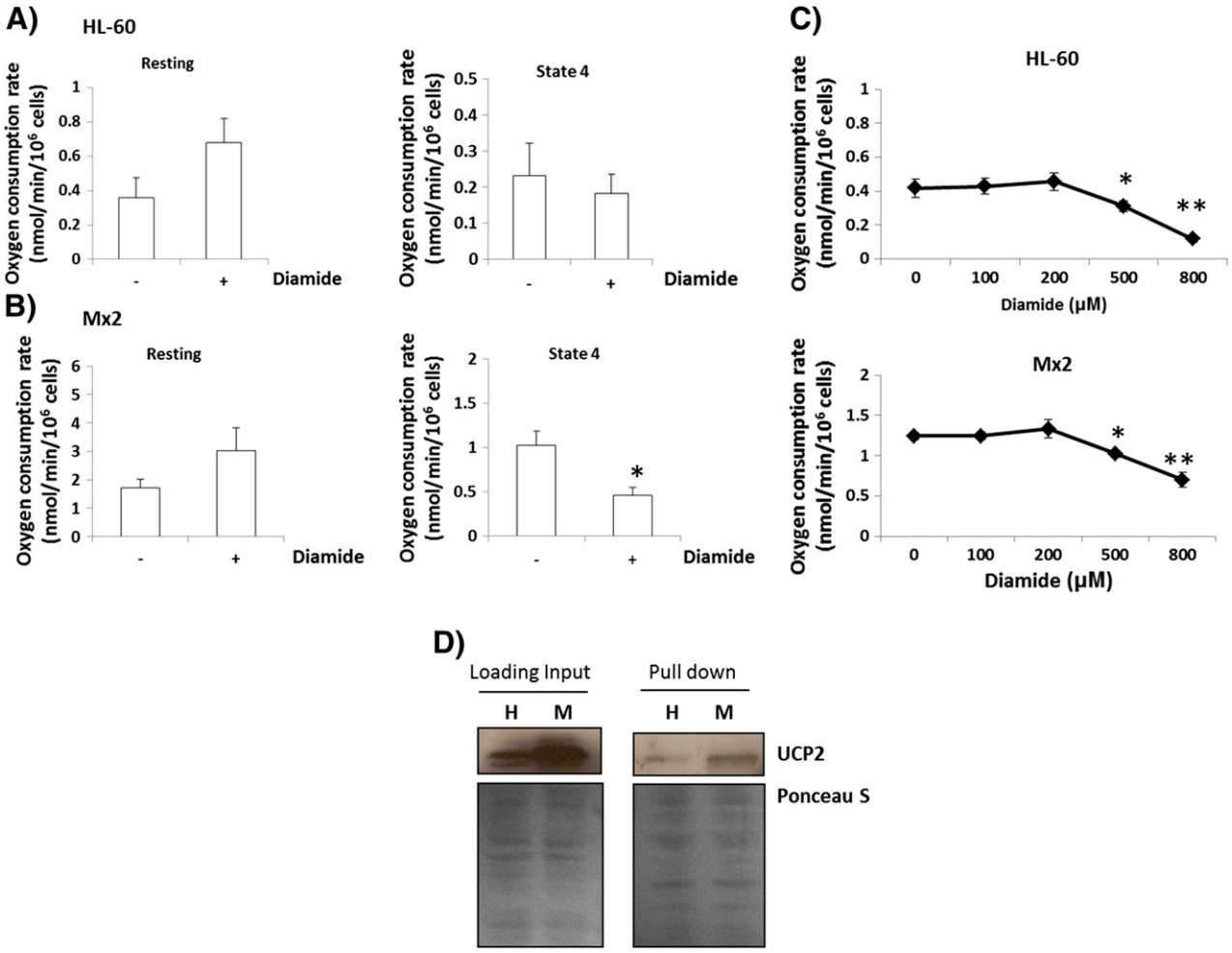

Fig. 3. Diamide selectively decreases proton leak-dependent respiration in drug resistant Mx2. Cells were exposed to 200 μM diamide and then tested for oxygen consumption under resting and state 4 conditions. Following the measurement of resting respiration, cells were treated with oligomycin (10 μg/mL) to induce state 4 respiratory conditions. (A) Resting and oligomycin-induced state 4 respiration in HL-60 cells. (B) Resting and oligomycin-induced state 4 respiration in Mx2 cells. $n=4$, Student's t test. * corresponds to $p \leq 0.05$. (C) Impact of diamide titration of resting respiration rates in HL-60 and Mx2 cells. Following the establishment of resting respiration rates diamide was titrated from 100 to 800 μM in the Clark-type electrode chamber. After diamide addition, rates were measured for up to 5 min. $n=4$, 1-way ANOVA with post-hoc Fisher test. * corresponds to $p \leq 0.05$, ** corresponds to $p \leq 0.01$. (D) Assessment of amount of glutathionylated UCP2 in HL-60 (H) and Mx2 (M) cells. Cells were treated with BioGEE, lysed, and then UCP2 was eluted using streptavidin beads. The amount of eluted UCP2 (pull down; PD) was detected by immunoblot. Loading input corresponds to the total amount of cell lysate used for UCP2 elution. Note that 60 μg of protein was used in the electrophoresis.

treated HL-60 and Mx2 cells with short hairpin UCP2 (shUCP2) lentiviral particles to knockdown UCP2 expression. Scrambled RNA served as the control. Transduction with shUCP2 led to ~76% and ~67% decrease in UCP2 protein levels in HL-60 and Mx2 (Fig. 4A). Next, we tested if knockdown of UCP2 in either cell type had any impact on resting or oligomycin-induced state 4 respiration and if the decreased amount of UCP2 abolished the diamide effect in the Mx2 cells. As shown in Fig. 4B, knockdown of UCP2 did not induce any changes in resting respiration in HL-60 cells. However, knockdown of UCP2 did induce a small decrease in oligomycin-induced (state 4; leak dependent) respiration (Fig. 4B). Pre-treatment with diamide had no effect on resting or oligomycin-induced respiration in HL-60 cells knocked-down for UCP2 (Fig. 4B). In contrast, knockdown of UCP2 in Mx2 cells induced a significant decrease in both resting and oligomycin-induced respiration (Fig. 4C). Moreover, knockdown of UCP2 abolished the diamide-mediated inhibition of this proton leak respiration in the Mx2 cells. It is also important to note that treatment of control Mx2 cells with diamide lowered proton leak to the same level as cells knocked down for UCP2. These results not only confirm that UCP2 plays a central role in mitochondrial energetics in drug resistant cells but that its activity can be suppressed with diamide. We also observed that UCP2 plays a small energetic role of drug sensitive HL-60 cells. However, UCP2 could not be inhibited in these cells by diamide. This could simply be due to the fact that HL-60 cells contain less UCP2 protein.

3.5. Diamide exposure sensitizes drug resistant Mx2 cells to chemotherapeutics

Mitochondria are now emerging as important targets for cancer treatment. For example, the chemotherapeutic cisplatin is known to induce its toxic effects by enhancing mitochondrial ROS production and inducing mitochondrial damage [21]. Proton leak through UCP2 or chemical induction of proton leak with protonophore FCCP mitigates these effects [21]. The targeted inhibition of UCP2 with drugs and natural products sensitizes drug resistant cancer cells to chemical agents [26,27]. Our observation that diamide acts as an inhibitor of UCP2 in drug resistant cells prompted us to decipher if this glutathionylation catalyst can sensitize the drug resistant Mx2 cells to chemotherapeutics. To do this, we employed the Biolog assay kit, which allows one to test the effects of a wide breadth of anti-cancer agents on cell status. Prior to the assay, HL-60 and Mx2 cells were pre-treated with or without 200 μM diamide (Fig. 5). Diamide pre-treatment sensitized HL-60 cells to two chemotherapeutic agents, namely, 4′-demethyl epipodophyllotoxin and

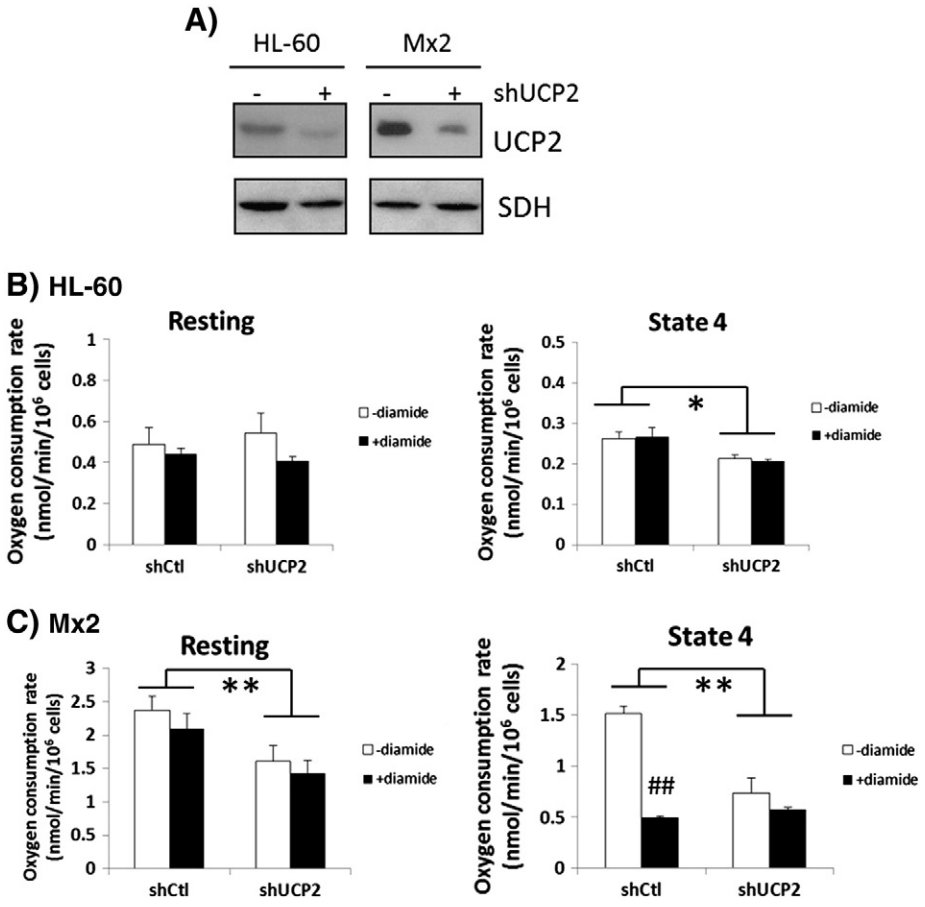

Fig. 4. Diamide inhibits leak through UCP2 specifically in Mx2 cells. HL-60 (H) and Mx2 (M) cells were treated with scrambled shRNA (control; shCtl) or shUCP2 lentiviral particles to knockdown UCP2. (A) Detection of UCP2 in cells treated with shCtl and shUCP2. (B) Resting and oligomycin-induced state 4 respiration in HL-60 cells treated or untreated with 200 μM diamide. (C) Resting and oligomycin-induced state 4 respiration in HL-60 cells treated or untreated with 200 μM diamide. $n = 4$, 2-way ANOVA with Fisher's post-hoc test. * corresponds to $p \leq 0.05$, ** corresponds to $p \leq 0.01$, shCtl vs. shUCP2. ## corresponds to $p \leq 0.01$, − diamide vs. + diamide.

13-*cis* retinoic acid. On the other hand, diamide pre-treatment sensitized Mx2 cells to six different chemotherapeutic agents including 4′-demethyl epipodophyllotoxin, Podofilox, all-*trans*-retinoic acid, mitomycin C, doxorubicin hydrochloride, and quercetin dihydrate (Fig. 5). It is interesting to note that diamide sensitized both HL-60 and Mx2 cells to 4′-demethyl epipodophyllotoxin which is a chemical analog of etoposide. Also, diamide sensitized Mx2 cells to doxorubicin but not daunorubicin which are both classified as anthracyclines. Although drugs have different sites of action, they can all induce oxidative stress through ROS production. This could be related to auto-catalysis of ROS production or the induction of ROS genesis by mitochondria (*e.g.*, through inhibition of the respiratory chain). For instance, all-*trans*-retinoic acid is known to induce ROS production [46]. Anthracyclines are able to auto-catalyze superoxide formation by cycling between oxidized and semi-reduced states [47]. The difference between doxorubicin and daunorubicin may be related to their ROS-generating properties. We had previously established that Mx2 cells have much lower levels of ROS in the presence of doxorubicin when compared to HL-60 cells. This difference was attributed to UCP2 function since inhibition of proton leak increased ROS levels and induced oxidative stress in Mx2 cells [26].

Our results from the Biolog assay indicate that diamide treatment sensitizes Mx2 cells to a variety of chemotherapeutics. The Biolog assay is based on the ability of cells to reduce a "redox dye" to a colored product that is detected spectrophotometrically. Reduction of the dye is reliant on the NADH generated by normal metabolic processes. As NADH levels are dependent on both production and consumption processes, it is not a direct measure of cell viability. Further, redox dyes like tetrazoliums can also react with NADPH which also increases in production when cells are exposed to ROS or ROS-generating drugs and molecules [48,49]. We thus decided to perform cell death and viability assays on diamide-treated cells exposed to either menadione or doxorubicin. We first tested if diamide-mediated inhibition sensitizes Mx2 cells to menadione, a naphthoquinone that produces superoxide. We had previously shown that UCP2 inhibition with genipin sensitizes Mx2 cells to menadione treatment [26]. Exposure of HL-60 cells to menadione (50 and 100 μM) for 24 h led to a robust increase in the amount of cell death (Fig. 6A). Diamide pre-treatment did not augment the toxicity of menadione in the HL-60 cells. In contrast, menadione did not induce any changes in Mx2 cell viability. However, co-treatment of Mx2 cells with diamide and menadione (50 and 100 μM) led to an increase in cell death (Fig. 6A). We next tested if diamide also increases the killing effect of doxorubicin. As shown in Fig. 6B, diamide did not enhance the toxicity of doxorubicin in HL-60 cells. On the other hand, diamide was able to increase the amount of Mx2 cell death following treatment with 0.1 and 0.5 μM doxorubicin (Fig. 6B). We then confirmed these observations by measuring the number of live cells using trypan blue. In the absence of diamide, menadione (50 and 100 μM) and doxorubicin (0.1 and 0.5 μM) reduced the number of live HL-60 cells in culture by ~50–60% (Fig. 7A and B). As expected, Mx2 cells displayed resistance to both menadione and doxorubicin treatment. Indeed, menadione treatment (50 and 100 μM) only reduced the number of live Mx2 cells by ~15%, whereas doxorubicin reduced live cell number by ~10% (0.5 μM) (Fig. 7A and B). However, diamide pretreatment enhanced the toxicity of menadione and doxorubicin in Mx2 but not HL-60 cells (Fig. 7A and B). This result would suggest that diamide disables the resistance of Mx2 cells to toxic agents. These observations are in full agreement with our previous work that

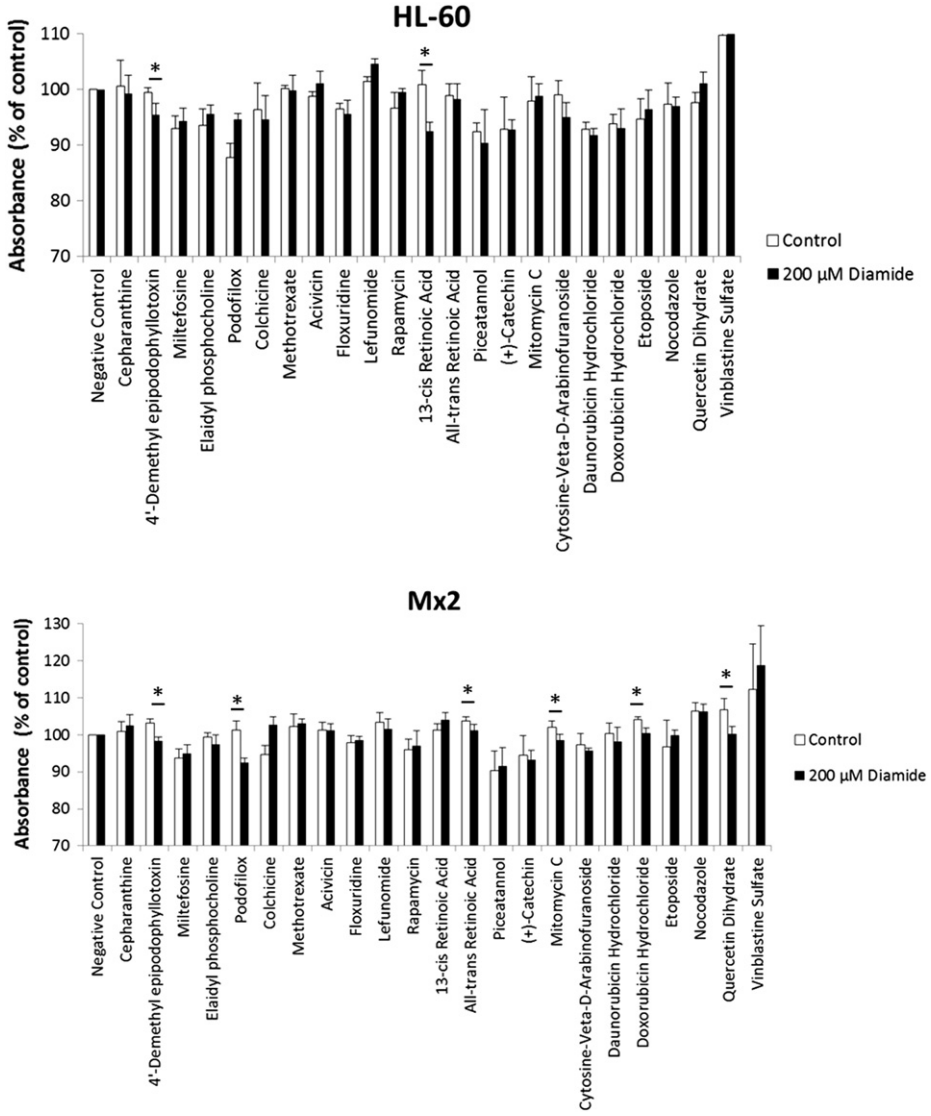

Fig. 5. Diamide treatment sensitizes Mx2 cells to various chemotherapeutics. Cells were treated or untreated with diamide (200 μM). Sensitivity to chemotherapeutics was tested using the Biolog assay (see Materials and methods). $n = 4$, Student's t test. * corresponds to $p \leq 0.05$.

illustrated that targeted inhibition of UCP2 with genipin sensitized Mx2 cells to anthracycline treatment [26]. Diamide pretreatment amplifies drug toxicity in Mx2 by deactivating proton leak through UCP2. This is achieved by the glutathionylation of UCP2. We have previously shown that the glutathionylation catalyst diamide deactivates leak through UCP2 by conjugating glutathione to exposed thiol residues [19]. Further, HL-60 cells do not express UCP2 in abundance unlike their drug resistant counterparts which use UCP2 to disable drug toxicity. Hence, diamide enhances drug toxicity by glutathionylating UCP2 in Mx2 cells.

Redox circuits are increasingly recognized as important in the modulation of protein function. To this end, glutathionylation serves a regulatory arm of redox signaling, modulating protein function through disulfide bridge formation between protein thiols and glutathione [50]. Glutathionylation is a highly regulated protein-specific mechanism that can however become deregulated under pathological conditions. Indeed, alterations in protein glutathionylation are associated with different cancer phenotypes and have been shown to play a role in drug resistance [51]. In this study, we showed that diamide-induced chemical glutathionylation of UCP2 in drug resistant Mx2 cells not only inhibits proton leak but sensitizes these cells to chemotherapeutics. Although diamide did reduce the amount of free glutathione in HL-60 and Mx2 cells, the diamide effects in Mx2 cells are attributed to UCP2 inhibition of proton leak by glutathionylation. These observations were confirmed by knocking down UCP2 in Mx2 cells and cross examining these effects using the drug sensitive parental HL-60 cell line. Manipulations in redox environment and the use of antioxidants have been considered as therapeutic strategies for cancer treatment [52,53]. However, such approaches do not work for all cancers, due to extreme variability in redox environment [54]. Here we have provided findings that could be exploited in the development of new approaches for the treatment of cancer. Further research is required to determine whether or not manipulation of glutathionylation can effectively be used in cancer cytotoxic strategies. Moreover a greater fundamental understanding of protein glutathionylation in oncology is necessary. However our findings indicate that the induction of UCP2 glutathionylation may serve as another tool in the eradication of cancer.

Acknowledgements

This research was funded through a grant from the Canadian Institutes of Health Research (CIHR), Institute of Nutrition, Metabolism and Diabetes (MEH) and a postdoctoral fellowship from CIHR (RJM).

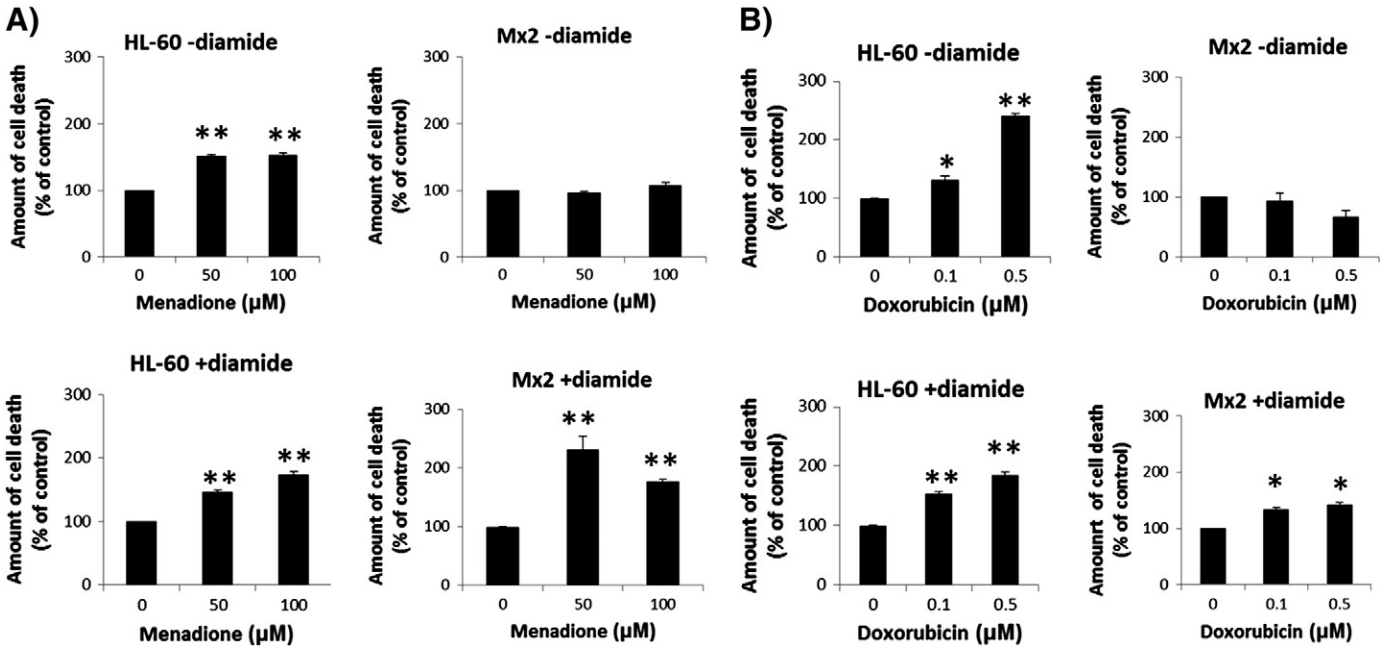

Fig. 6. Measurement of amount of cell death by PI assay. (A) Impact of diamide on menadione toxicity. HL-60 and Mx2 cells were treated or untreated with diamide for 30 min and then incubated for 24 h with menadione (0–100 μM). (B) Effect of diamide on doxorubicin toxicity. HL-60 and Mx2 cells were treated or untreated with diamide for 30 min and then incubated for 24 h in doxorubicin (0–0.5 μM). $n=4$, 1-way ANOVA with Fisher's post-hoc test. * corresponds to $p\leq 0.05$, ** corresponds to $p\leq 0.01$.

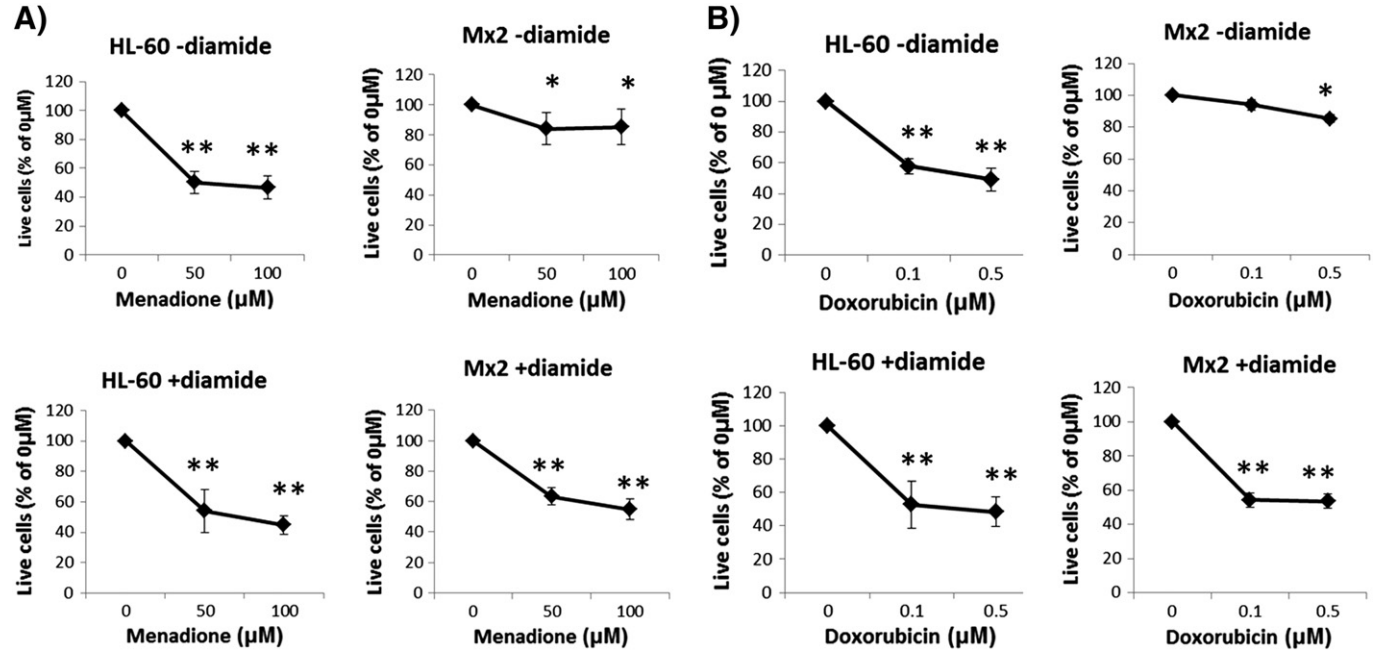

Fig. 7. Measurement of amount of live cells by trypan blue exclusion assay. (A) Impact of diamide on menadione toxicity. HL-60 and Mx2 cells were treated or untreated with diamide for 30 min and then incubated for 24 h with menadione (0–100 μM). (B) Effect of diamide on doxorubicin toxicity. HL-60 and Mx2 cells were treated or untreated with diamide for 30 min and then incubated for 24 h in doxorubicin (0–0.5 μM). $n=4$, 1-way ANOVA with Fisher's post-hoc test. * corresponds to $p\leq 0.05$, ** corresponds to $p\leq 0.01$.

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